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(54) **Title:** CONDITIONAL SUICIDE CELLS OF E. COLI ETC.

(57) **Abstract**

The potential risks associated with the intentional or unintentional release of genetically engineered microorganisms led to the construction of biological containment systems by which bacteria are killed in a controlled suicide process. In previously published suicide systems cell killing was caused by proteins destroying the cell membrane or cell wall. Here a conditional cell-killing system is presented which is based on the intracellular degradation of cellular DNA. The nuclease gene used was that of the extracellular nuclease of *Serratia marcescens*. The nuclease gene was deleted for the leader-coding sequence and the truncated gene was put under the control of the lambda P_L promoter. Following thermoinduction of the nuclease gene cassette in *Escherichia coli*, the cell survival dropped to 2×10^{-5}, and more than 80 % of the radioactivity labelled DNA was converted to acid soluble material within 2.5 h in the absence of cell lysis. Cells from the majority (84 %) of clones which survived thermoinduced killing turned out to be as sensitive to a second thermoinduction as the original strain. Cells of the other clones showed a somewhat slower killing kinetics or a slightly higher final level of survivors. The suicide system described combines the regulated killing of cells with the abortion of horizontal gene transfer processes by destroying DNA otherwise potentially available for conjugation, transduction and genetic transformation.

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Conditional suicide cells of E. coli etc.

The use of genetically engineered microorganisms (GEMs) in closed systems bears the possibility of accidental releases of the cells into the environment. Moreover, the planned and the already performed applications of GEMs in agriculture, waste treatment, and production of certain raw materials rely on the release of great quantities of cells into the environment. A main concern about releases of transgenic organisms comes from the uncertainty on how these organisms will behave in the environment (30). One aspect of this is the risk of unanticipated survival and reproduction in the environment with negative ecological effects. Another problem is seen in the uncontrolled transfer of the genetically engineered DNA to other organisms in the environment. Among bacteria, such horizontal gene transfer can proceed by various processes such as conjugation, transduction and transformation in natural microbial habitats (15, for review see reference 28). Therefore, as a biological containment strategy, conditional suicide systems for bacteria have been designed and constructed which cause the controlled death of the cells (for review see reference 20). Such suicide systems consist of a regulated killing gene which is expressed in response to specific factors in the environment or in closed systems. The killing genes so far successfully used in suicide systems determined the synthesis of cell-lysing agents including membrane-destabilizing polypeptides (7, 14, 19), cell-destroying levane (23) and lysozyme (27). The genes were put under the control of promoters inducible by e.g. increased temperature, starvation

or the presence of certain chemicals (11, 19, 27). However, the use of membrane destructing agents for killing of cells would possibly faciliate the release of recombinant DNA into the surrounding milieu. Since experimental data suggest that free DNA can persist, e.g., in nonsterile soils for periods of weeks or months (24, 25) and is available for uptake by bacterial cells that are naturally transformable, it is assumed that released DNA can be disseminated among bacteria in the environment by genetic transformation (16). Therefore, a bacterial suicide system combining controlled killing of cells with the destruction of genetic material before its release from cells would be desirable in order to limit both the survival of GEMs and the transfer of the recombinant DNA to other organisms.

An object of the invention is to provide a conditional suicide system by which cell death is accompanied by a degradation of intracellular DNA to acid soluble material.

The invention provides conditional suicide cells of *Escherichia coli* genetically engineered and comprising

(a) a first plasmid comprising
- a promoter
- under the control of a temperature sensitive repressor at ambient temperature but not at elevated temperatures, and
- downstream of the promoter, a nuclease gene which can be expressed in the cells by means of said promoter, and

(b) optionally another plasmid for the expression of a structural gene other than the nuclease gene.

Further, the invention provides conditional suicide cells of *Escherichia coli* genetically engineered and comprising a plasmid comprising

a) a first promoter under the control of a temperature sensitive repressor at ambient temperature but not at elevated temperatures; and downstream of the promoter, a nuclease gene which can be expressed in the cells by means of said promoter; and

(b) optionally another promoter and downstream of the promoter a structural gene other than the nuclease gene, the structural gene being expressable by means of said other promoter.

Further, the invention provides cells according to claim 1 or 2, characterized in that the cells can express altogether two or more nuclease genes.

Further, the invention provides conditional suicide cells according to any of the preceeding claims, characterized by the gene for the nuclease of *Serratia marcescens* deleted for the leader-coding sequence as said nuclease gene, where the nuclease gene is that of the wild type or a variant thereof which still kills cells of *Escherichia coli* and degrades intracellularly their DNA.

Further, the invention provides conditional suicide cells according to any of the preceeding claims, characterized by the lambda P_L-promoter and/or the temperature sensitive repressor of phage lambda.

Finally, the invention provides plasmid pAH12 as plasmid according to claim 1(a) or claim 2(a), obtainable from plasmid pNuc4 by

(a) cutting out the nuc gene without the leader-coding sequence and inserting said gene into vector pET81F$^+$,

(b) cutting out the nuc gene together with the ribosome binding site and/or the T7Phi10 promoter and the start codon from the plasmid resulting from step (a) and inserting said fragment downstream of the P_L promoter of plasmid pSF1 (after deletion of the ssb gene) and

(c) isolating the product as plasmid pAH12.

FIG. 1. Construction of the containment plasmid pAH12 (for details refer to 'Results').

L: nucleotide sequence of the leader peptide, *nuc*: coding sequence for the mature *Serratia* nuclease, Φ10: T7 Φ10 promoter, P_L: lambda P_L promoter, boxes: vector DNA, line: insert DNA, ATG: start codon, arrowheads: indicate location and direction of promoters.

FIG. 2. Survival of *E. coli* TGE900 with the suicide plasmid pAH12 (■) or the vector pSF1E (□) after thermoinduction by incubation at 42 °C (0 to 40 min). Viable counts were determined as described in 'Materials and Methods'. The survival (N/N_0) is the viable count of the culture at the indicated times (N) divided by the viable count of the culture before induction (N_0). The data are means of two (pSF1E) or three (pAH12) independent experiments.

FIG. 3. Intracellular DNA degradation in *E. coli* TGE900 pAH12 following thermoinduction by incubation at 42 °C (0 to 30 min). The cells were labeled with [^{3}H]thymidine at 28 °C and treated as described in 'Materials and Methods'. At the indicated times the TCA-insoluble radioactive material was determined in the thermoinduced culture (■), in the culture thermoinduced in the presence of Cm (▽) and in the culture kept at 28 °C throughout (○).

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 contains the description of bacterial strains and plasmids used in this study. *Escherichia coli* AH1 was constructed by P1 transduction (18) using *E. coli* JC10289 pKY102 as donor of the *recA* deletion (12).

Molecular techniques. Plasmid DNA was isolated by alkaline lysis (6) for treatment with restriction endonucleases and for transformations. To inhibit the extracellular nuclease of *S. marcescens* (in the following termed *Serratia* nuclease) during plasmid preparation the method of Birnboim and Doly (6) was modified. Before lysis the cells were washed in 0.5 ml 10 mM NaCl to remove extracellular nuclease. During the alkaline lysis incubation times were shortened to 5 min and all centrifugations were done at 4 °C. After the first precipitation of the DNA with ethanol, the pellet was resuspended in 50 µl 10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0. After addition of 25 µl 7.5 M NH_4-acetate, pH 7.5 the mixture was incubated for 15 min at 70°C, for 10 min on ice and then centrifuged (12 min, 13.000 x g, 4°C). The supernatant was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1), once with chloroform/isoamylalcohol (24:1) and precipitated with ethanol. The pellet was washed with ice-cold 70 % ethanol, dried and resuspended in 10 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0). For cloning experiments the plasmid DNA was isolated with the Qiaprep-spin plasmid kit

(Diagen, Düsseldorf, Germany) and extracted twice with phenol/chloroform/isoamylalcohol as described above.

Cloning experiments were done by standard procedures (17). *E. coli* was transformed by electroporation (Gene pulser, Bio-Rad, Munich, Germany; 25 μF, 12.5 kV cm $^{-1}$, 200 Ohm)'.

Determination of survival after thermoinduction of killing. Cells were grown in Luria-Bertani (LB) broth (9) supplemented with ampicillin (Ap, 100 μg ml^{-1}) at 28 °C to approximately 2 x 10^8 cells ml^{-1} (log-phase cells). The culture was divided into two equal parts. One part was further incubated at 28 °C, the other part was incubated at 42 °C for the thermoinduction of the lambda P_L promoter. After various time intervals viable counts were determined by serial dilution and plating on LB agar with Ap (100 μg ml^{-1}). Plates were incubated for 24 h at 28 °C. The survival (N/N_0) is the viable count of the culture after the indicated times (N) divided by the viable count of the culture before induction (N_0).

Intracellular DNA degradation. DNA was labeled by growth of the cells in LB broth plus Ap (100 μg ml^{-1}), 2'deoxyadenosine (250 μg ml^{-1}) and [*methyl*-^{3}H]thymidine (1.5 x 10^5 Bq ml^{-1}) for three generations at 28 °C. At about 2 x 10^8 cells ml^{-1} the cells were collected by filtration, washed in 30 ml prewarmed phosphate buffer and suspended in 30 ml prewarmed LB broth plus Ap (100 μg ml^{-1}). The culture was divided into three equal parts and incubated further at 28 °C. After 30 min one culture was supplemented with chloramphenicol (Cm, 100 μg ml^{-1}) to inhibit protein expression and was incubated for 30 min at 42

°C for thermoinduction of the lambda P_L promoter. The second culture was treated the same except that Cm was omitted. After thermoinduction incubation of the two cultures was continued at 28 °C. The third culture was incubated at 28 °C throughout the experiment. After various time periods 0.2 ml-samples of the three cultures were removed and the cells were precipitated by addition of 0.2 ml of ice-cold 12 % trichloroacetic acid (TCA). After incubation on ice for at least 30 min the precipitate was collected on glass-fiber filters (GF102; Schleicher & Schuell, Dassel, Germany), washed with 3 ml of ice-cold TCA and then rinsed twice for 2 min in baths containing 6 % TCA. Then the filters were rinsed in ice-cold 70 % ethanol and dried at 40 °C for 14 h. Filters were placed into 10 ml of toluene-based scintillation fluid (Unisolve 1; Werner Zinsser Scintillators, Frankfurt, Germany), and TCA-insoluble radioactivity was determined in a Betamatic scintillation counter (Kontron, Eding, Germany).

RESULTS

Construction of the suicide cassette. The *nuc* gene coding for the *Serratia* nuclease determines a polypeptide of 266 amino acids of which the N-terminal 21 amino acids constitute a leader peptide (5). The leader peptide is removed during secretion which activates the mature nuclease (2, 5). The construction of a containment plasmid (pAH12) is described in Fig. 1. The plasmid contains the nuclease gene of *S. marcescens* deleted for the leader-coding sequence under the control of the lambda P_L promoter. In *E. coli* TGE900 (8) which carries the gene for the thermosensitive lambda cI857 repressor in the chromosome the expression of the truncated gene for the leader-free *Serratia* nuclease is controlled by temperature.

The DNA fragment coding for the mature *Serratia* nuclease was cut out from the plasmid pNuc4 (2) by digestion with *Eag*I and *Bss*HII. After mung bean nuclease digestion the fragment was joined to a start codon in the vector pET81F$^+$ (29). pET81F$^+$ was prepared for cloning by digestion with *Nco*I and *Bam*HI and filling in of the resulting single-stranded ends of the vector with the Klenow fragment of DNA polymerase I of *E. coli*. The resulting plasmid was termed pAH10 (Fig. 1). In a second step the truncated nuclease gene together with the ribosome-binding site and the start codon of pET81F$^+$ was cloned downstream of the lambda P_L promoter of the pBR322 derived plasmid pSF1 (4) by blunt end ligation. For this pAH10 was digested with *Ssp*1, *Bam*HI and mung bean nuclease and pSF1 with *Eco*RI and mung bean nuclease. The ligation mixture was transformed into *E. coli* TGE900. To screen for transformants which contained an active

suicide cassette, clones obtained at 28 °C were streaked on LB agar plus Ap (100 μg ml^{-1}) and incubated at 28 °C, and for thermoinduction of the lambda P_L promoter at 42 °C. Clones having an active suicide cassette grew only at 28 °C incubation. Among 40 Ap-resistant transformants screened 12 were thermosensitive for growth. From the thermosensitive clones plasmid DNA was isolated and analyzed by restriction mapping. One plasmid which had the inserted nuclease cassette in the correct orientation relative to the P_L promoter was termed pAH12 (Fig. 1).

Thermoinduction of killing. Log-phase broth cultures of *E. coli* TGE900 with pAH12 or the vector pSF1E grown at 28 °C were shifted from 28 °C to 42 °C. At 42 °C the thermosensitive lambda cI857 repressor is rapidly inactivated thereby derepressing the transcription of the nuclease gene from the lambda P_L promoter. In a time course experiment (Fig. 2) the survival of cells was determined by plating at 28 °C on LB agar with Ap following the heat pulse. The survival of *E. coli* TGE900 with pAH12 but not with the vector plasmid (control) declined rapidly after thermoinduction and reached its minimum after 30 min (2.3×10^{-5}). A series of additional experiments (Table 2) led to the conclusion that the cell killing resulted specifically from the derepression of the nuclease gene in strain TGE900 pAH12. First, thermoinduction of TGE900 with the vector pSF1E did not substantially affect growth during the 30 min induction period compared to the 28 °C culture. Secondly, when the vector contained the *ssb* gene of *E. coli* coding for single-stranded DNA binding protein, the thermoinducted

overproduction of this protein did not cause killing either. This suggests that killing of cells with pAH12 by thermal treatment did not result merely by derepression of an additional protein-coding gene on the plasmid. The slight decrease of survival following thermoinduced overproduction of *ssb* protein (Table 2) is in accord with a previous observation and probably results from interference of excess *ssb* protein with replication (22). Finally, a *recA* deletion was crossed into TGE900 pAH12 (giving AH1 pAH12) to examine whether reduced DNA repair capacity of the cells would disclose chromosomal DNA damage caused by low levels of *Serratia* nuclease expressed even in uninduced cells. This could result in a lower growth rate and increased sensitivity to thermoinduction. This, however, was not the case. The reduced growth of AH1 pAH12 at 28 °C (Table 2) was also observed with AH1 containing the vector pSF1E (not shown) and is typical for *recA* mutants compared to their *recA*$^+$ counterparts. The thermoinduced killing was not enhanced in the *recA* derivative (Table 2). Thus, nuclease expression from pAH12 in TGE900 appears to be very low or absent at 28 °C. This conclusion is also supported by the observation that under the condition of repression of the P_L promoter (28 °C) there is no difference in the growth rates of *E. coli* TGE900 with the plasmids pAH12 (nuc), pSF1E (vector) or pSF1 (*ssb*) (Table 2). The similar level of killing of TGE900 pAH12 and its *recA* counterpart AH1 pAH12 upon thermoinduction (Table 2) suggests that the repair capacity of the cells does not help against the killing effect of the derepressed nuclease.

Examination of surviving clones. After thermoinduction of killing survival occured at a low but significant level. Since the viable counts were determined by plating on LB agar with Ap the growth of plasmid free segregants was excluded as a cause for survival. The phenotypes and genotypes of surviving clones were examined in order to find out whether the survival resulted from a "transient resistance" or a mutation. A mutation could be located in the plasmid pAH12 (e.g. in the promoter/operator region or in the *nuc* gene) or in the bacterial chromosome (e.g. in the cI857 repressor gene). Surviving clones were streaked on LB agar with Ap and incubated at 28 °C and at 42 °C using survivors from the 28 °C plate. This procedure was repeated three times. Among 25 examined clones 21 behaved like the wildtype *E. coli* TGE900 pAH12, i.e., they fully grew at 28 °C, but only few colonies appeared at 42 °C in the streaks. It was concluded that these survivors of a thermoinduction (84 %) are not resistant against the intracellular action of the *Serratia* nuclease or that they are not *nuc* defective mutants. In streaks of four clones more colonies appeared at 42 °C than in streaks of TGE900 pAH12. To see whether these four clones had an altered plasmid or a chromosomal mutation, *E. coli* TGE900 was transformed with plasmid DNA isolated from the four clones. The transformants showed the same growth in streaks on LB agar with Ap at 42 °C as the original surviving clones, suggesting that chromosomal mutations were not responsible for the survival of the clones. Gel electrophoretic analysis of the DNA of the four plasmid clones showed the same plasmid size as that of the wildtype plasmid pAH12. Therefore, the presence of large deletions in

the plasmids can be ruled out, but not of small deletions or point mutations. From the four original clones and the four secondary clones the time course of killing at 42 °C in log cultures was determined (Table 3). The killing of original and secondary clones was similar. Three of the four clones (# 8, 13, 16) showed a slower killing kinetics than TGE900 pAH12 (Fig. 2) but reached a similar low level of survival as strain TGE900 pAH12 (Fig. 2) after 60 min of incubation at 42 °C. One clone showed a reduced killing (survival of approximately 7 x 10^{-4}) compared to the wildtype (2.3 x 10^{-5}, see Table 2). In summary, 21 of the examined 25 surviving clones were only transiently refractory to thermoinduced killing. Possibly the P_L promoter was not induced in these cells during the first heat treatment. The other 4 surviving clones showed a slightly decreased sensitivity to heat treatment resulting in a somewhat slowed down killing kinetics which is possibly due to mutations located on the plasmid. In none of the 25 clones thermoinduced killing was totally aborted.

Intracellular DNA degradation. The extracellular *Serratia* nuclease is an endonuclease and degrades high molecular weight DNA to acid soluble material (21). Previous studies had revealed that the nuclease introduces single- and double-strand breaks into duplex DNA which eventually leads to the breakdown of DNA into oligo- and mononucleotides (1). The thermoinduction of the gene coding for the leader-free *Serratia* nuclease in *E. coli TGE900* pAH12 led to the breakdown of intracellular (chromosomal and plasmid) DNA (Fig. 3). In the experiments, the cellular DNA was labeled with [^{3}H]thymidine and the fraction of

TCA-insoluble radioactivity was determined after various incubation times. Following induction for 30 min at 42 °C DNA was degraded to acid soluble material for at least 2.5 h during which cell lysis did not occur. The data show that the *Serratia* nuclease without the leader peptide is active in the cytoplasm of the cells. DNA degradation (and possibly RNA degradation) continued over a period of at least 3 h after derepression of the truncated *nuc* gene leading to the conversion of over 80 % of cellular DNA to acid soluble material. In cells grown at 28 °C all the time or thermoinduced at 42 °C with simultaneous addition of Cm (100 μg ml^{-1}), there was little if any DNA degradation.

DISCUSSION

The suicide system presented here consists of the truncated *nuc* gene of *Serratia marcescens* (deletion of the sequence for a leader peptide) coding for a powerful DNase and RNase cloned downstream of the lambda P_L promoter and controlled by the thermosensitive lambda cI857 repressor. Upon thermoinduction, cell killing correlated with DNA degradation, i.e. thermoinduction (30 min 42 °C) led to a minimum of cell survival and to extensive intracellular DNA breakdown. In the system the expression of the nuclease gene is limited to the period before the coding sequences are destroyed by the enzyme. Nevertheless, the amount of enzyme produced following induction suffices possibly together with other cellular DNases to degrade the majority of intracellular DNA in the culture to acid soluble material within 3 h. The intracellular milieu apparently does not only provide favorable conditions for the activity of the enzyme but also may contribute to the stability of the enzyme by providing a high concentration of proteins which was shown to stimulate the nuclease activity towards RNA and DNA in vitro and to stabilize the purified enzyme against thermal inactivation (1). The intracellular activity of the enzyme is notable, because the *Serratia* nuclease has two disulfide bonds, which are essential for the activity of the extracellular enzyme (3), and the formation of which could be inhibited under the prevailing redox conditions in the intracellular milieu. Recently it was found that the overexpression of the *nuc* gene with the leader peptide-coding sequence in *E. coli* resulted in aggregation and sequestration

of the protein in inclusion bodies, from which active leader-free *Serratia* nuclease could be received by in vitro procedures (10).

The efficiency of killing by the system (2×10^{-5}) is similar to or better than that of previously published suicide systems not involving a nuclease. For example, efficiencies of 5×10^{-2} (26), 10^{-3} (23), 10^{-5} to 10^{-6} (7, 13) and 10^{-8} by using two copies of the killing gene (13) were described. The fact that out of 25 examined survivors of a thermoinduction most were as sensitive as the initial strain and the rest was still highly sensitive suggests that the efficiency of the system is not very prone to mutational escape events. Knudsen and Karlström (14) described a suicide system based on the *relF* gene of *E. coli* controlled by various *lac* promoters localized on plasmids. The authors found that surviving clones contained plasmids with mutations in the suicide cassette. The efficiency of the suicide system could be increased by duplication of the suicide cassette. In another suicide system (7) with the *gef* gene product as killing function, 31 survivors were examined, from which 21 (68 %) were Gef resistant. The two plasmids carrying the killing and regulatory elements seemed to be unchanged so that the host mechanism to escape killing remained unknown.

The nucleolytic killing of cells is assumed to prevent horizontal gene transfer effectively. Cells with degraded DNA cannot function as donors in conjugation, transduction or transformation. Since cell lysis did not occur following induction of killing, even large DNA fragments produced during initial stages of DNA degradation do not normally enter the environment where transformation of other cells could occur.

Also, it seems unlikely that cells containing DNase (and degraded DNA) will preserve foreign DNA when acting as recipient.

The suicide system based on the controlled expression of a nucleotide sequence-independent nuclease can be adopted for a variety of applications. For instance, for the regulation of cell survival in the environment the nuclease killing gene could be put under the control of other regulators more relevant to the environment, such as those responding to starvation for specific substances. For use in closed systems the physiologically induced suicide of cells could substitute for killing by addition of chemicals. Moreover, since cell lysis does not accompany killing, during industrial production of substances by GEMs the cell entity is preserved while the mass of DNA (and possibly RNA) is degraded leading to reduced efforts needed to remove nucleic acids from the product. For such applications a chemical induction of the truncated nuclease gene expression could be desirable (such as IPTG) if high temperature regimes would have to be avoided due to a thermal sensitivity of the product.

TABLE 1. Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
E. coli TGE900	F', *sup*$^+$, *ilv*, *bio*(λcI857ΔB*am*ΔHI)	8
E. coli AH1	TGE900 Δ(*recA-srl*) 306::Tn10, Tc^R	This work
pET81F$^+$	Ap^R, 2730 bp; expression vector with the T7 Φ10 promoter, ribosome binding site and ATG start codon	29
pSF1	Ap^R, 4820 bp; contains the *ssb* gene of *E. coli* under control of the lambda P_L promoter	4
pSF1E	Ap^R, 4149 bp; pSF1 was digested with *Eco*RI for deletion of the *ssb* gene and religated	This work
pNuc4	Ap^R, 4128 bp; gene for the extracellular nuclease of *Serratia marcescens* SM6 (*nuc*) in vector pUC18	2
pAH10	Ap^R, 3660 bp; leader-free nuclease gene of *S. marcescens* under control of the T7 Φ10 promoter in vector pET81F$^+$	This work
pAH12	Ap^R, 5227 bp; leader-free nuclease gene of *S. marcescens* under control of the lambda P_L promoter in vector pSF1E	This work

TABLE 2. Survival of *E. coli* strains at 28 °C and after thermoinduction of the lambda P_L promoter (42 °C).

Strain	Time at 42 °C or 28 °C (min)	Survival (N/N_0)[a]	
		42°C-culture	28°C-culture
TGE900 pAH12 (*nuc*)	0	1	1
	30	2.3×10^{-5}	1.5
TGE900 pSF1E (vector)	0	1	1
	30	1.2	1.5
TGE900 pSF1 (*ssb*)	0	1	1
	30	0.8	1.5
AH1 pAH12 (*nuc*, *recA*)	0	1	1
	30	5.8×10^{-5}	1.1

[a] The survival (N/N_0) is the viable count of the culture at the indicated times (N) divided by the viable count of the culture before induction (N_0; for details, refer to 'Materials and Methods'). The data are means of two independent experiments.

TABLE 3. Thermoinduced killing of *E. coli* TGE900 pAH12 clones which had survived a 42 °C treatment[a]

TGE900 pAH12 mutant number	Phenotype[b]	Induction time at 42 °C (min)	Survival (N/No)
8	slightly	0	1
	resistant	30	1×10^{-5}
		60	8×10^{-6}
8[c]	slightly	0	1
	resistant	30	2×10^{-4}
		60	8×10^{-6}
13	slightly	0	1
	resistant	30	8×10^{-5}
		60	6×10^{-5}
13[c]	slightly	0	1
	resistant	30	4×10^{-5}
		60	$\leq 4 \times 10^{-5}$
16	slightly	0	1
	resistant	30	9×10^{-4}
		60	2×10^{-5}
16[c]	slightly	0	1
	resistant	30	3×10^{-4}
		60	2×10^{-5}
18	medium	0	1
	resistant	30	7×10^{-4}
		60	7×10^{-4}
18[c]	medium	0	1
	resistant	30	2×10^{-3}
		60	$\leq 3 \times 10^{-4}$

[a] Log-phase cells of *E. coli* TGE900 pAH12 were thermoinduced as described in 'Materials and Methods' and survivors after 30 min at 42 °C were obtained by plating on LB agar plus Ap (100 μg ml^{-1}) and incubation at 28 °C. Log-phase cells from such surviving clones were again thermoinduced at 42 °C for 30 and 60 min.

[b] Classification after incubation at 42 °C on agar plates (cells were streaked on LB agar with Ap)

[c] The plasmid DNA from the original clone was isolated and used to transform *E. coli* TGE900.

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Patent Claims

1. Conditional suicide cells of *Escherichia coli* genetically engineered and comprising

(a) a first plasmid comprising
- a promoter
- under the control of a temperature sensitive repressor at ambient temperature but not at elevated temperatures, and
- downstream of the promoter, a nuclease gene which can be expressed in the cells by means of said promoter, and

(b) optionally another plasmid for the expression of a structural gene other than the nuclease gene.

2. Conditional suicide cells of *Escherichia coli* genetically engineered and comprising a plasmid comprising

a) a first promoter under the control of a temperature sensitive repressor at ambient temperature but not at elevated temperatures; and downstream of the promoter, a nuclease gene

which can be expressed in the cells by means of said promoter; and

(b) optionally another promoter and downstream of the promoter a structural gene other than the nuclease gene, the structural gene being expressable by means of said other promoter.

3. Cells according to claim 1 or 2, **characterized** in that the cells can express altogether two or more nuclease genes.

4. Conditional suicide cells according to any of the preceeding claims, **characterized** by the gene for the nuclease of *Serratia marcescens* deleted for the leader-coding sequence as said nuclease gene, where the nuclease gene is that of the wild type or a variant thereof which still kills cells of *Escherichia coli* and degrades intracellularly their DNA.

5. Conditional suicide cells according to any of the preceeding claims, **characterized** by the lambda P_L-promoter and/or the temperature sensitive repressor of phage lambda.

6. Plasmid pAH12 as plasmid according to claim 1(a) or claim 2(a), obtainable from plasmid pNuc4 by

(a) cutting out the nuc gene without the leader-coding sequence and inserting said gene into vector pET81F$^+$,

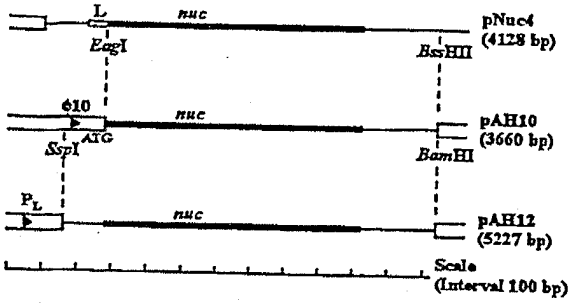
(b) cutting out the nuc gene together with the ribosome binding site and/or the T7Phi10 promoter and the start codon from the plasmid resulting from step (a) and inserting said fragment downstream of the P_L promoter of plasmid pSF1 (after deletion of the ssb gene) and

(c) isolating the product as plasmid pAH12.

SUMMARY

The potential risks associated with the intentional or unintentional release of genetically engineered microorganisms led to the construction of biological containment systems by which bacteria are killed in a controlled suicide process. In previously published suicide systems cell killing was caused by proteins destroying the cell membrane or cell wall. Here a conditional cell-killing system is presented which is based on the intracellular degradation of cellular DNA. The nuclease gene used was that of the extracellular nuclease of *Serratia marcescens*. The nuclease gene was deleted for the leader-coding sequence and the truncated gene was put under the control of the lambda P_L promoter. Following thermoinduction of the nuclease gene cassette in *Escherichia coli*, the cell survival dropped to 2×10^{-5}, and more than 80 % of the radioactivly labeled DNA was converted to acid soluble material within 2.5 h in the absence of cell lysis. Cells from the majority (84 %) of clones which survived thermoinduced killing turned out to be as sensitive to a second thermoinduction as the original strain. Cells of the other clones showed a somewhat slower killing kinetics or a slightly higher final level of survivors. The suicide system described combines the regulated killing of cells with the abortion of horizontal gene transfer processes by destroying DNA otherwise potentially available for conjugation, transduction and genetic transformation.

Fig. 1



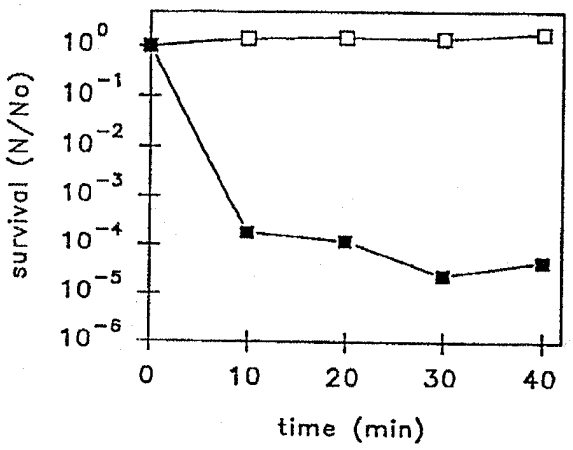


Fig. 2

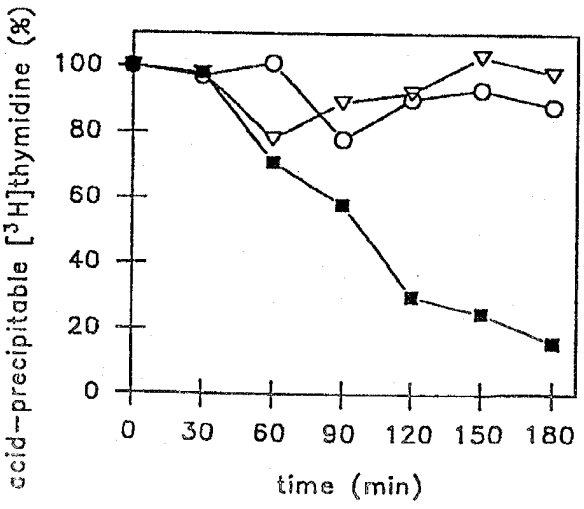


Fig. 3

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/02245

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/22 C12N15/64 C12N15/70 //C12N1/21,C12R1:19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNUAL REVIEW OF MICROBIOL., vol. 47, 1993 ANNUAL REVIEWS INC. PALO ALTO, CALIFORNIA, US, pages 139-166, S. MOLIN ET AL. 'Suicidal genetic elements an dtheir use in biological containment of bacteria' cited in the application see page 152, line 36 - page 153, line 9 see page 148, line 17 - page 150, line 2 --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1995	10.10.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Herrmann, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	APPLIED MICROBIOL. AND BIOTECHNOL., vol. 59, no. 5, May 1993 SPRINGER INTERNATIONAL, NEW YORK, US, pages 1361-1366, G. RECORBET ET AL. 'Conditional suicide system of Escherichia coli released into soil thet uses the bacillus subtilis sacB gene' cited in the application the whole document	1-6
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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 95/02245

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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